

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : C07K 15/00, 3/20, 3/28 C12P 21/00	A1	(11) International Publication Number: WO 88/ 01624 (43) International Publication Date: 10 March 1988 (10.03.88)
(21) International Application Number: PCT/US87/02140 (22) International Filing Date: 27 August 1987 (27.08.87) (31) Priority Application Number: 908,426 (32) Priority Date: 4 September 1986 (04.09.86) (33) Priority Country: US (71) Applicant: IMMUNEX CORPORATION [US/US]; 51 University Street, Seattle, WA 98101 (US). (72) Inventor: KRONHEIM, Shirley, R. ; 11526 36th Street, NE, Seattle, WA 98125 (US). (74) Agent: BUTLER, F., Prince; Griffin, Branigan & Butler, Box 2326, Arlington, VA 22202 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: PURIFICATION OF RECOMBINANT INTERLEUKIN-1 (57) Abstract A process for purifying recombinant IL-1 from microbial cells, comprising suspending the cells in an aqueous buffered medium having a pH from about 1 to about 5; disrupting the cells to provide an extract containing solubilized IL-1; and recovering solubilized IL-1 from the extract.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT Austria	FR France	ML Mali
AU Australia	GA Gabon	MR Mauritania
BB Barbados	GB United Kingdom	MW Malawi
BE Belgium	HU Hungary	NL Netherlands
BG Bulgaria	IT Italy	NO Norway
BJ Benin	JP Japan	RO Romania
BR Brazil	KP Democratic People's Republic	SD Sudan
CF Central African Republic	of Korea	SE Sweden
CG Congo	KR Republic of Korea	SN Senegal
CH Switzerland	LI Liechtenstein	SU Soviet Union
CM Cameroon	LK Sri Lanka	TD Chad
DE Germany, Federal Republic of	LU Luxembourg	TG Togo
DK Denmark	MC Monaco	US United States of America
FI Finland	MG Madagascar	

5

TITLE

10

Purification of Recombinant
Interleukin-1BACKGROUND OF THE INVENTION

The present invention relates generally to protein chemistry, and specifically to processes for purifying recombinant proteins produced by high level expression in microorganisms.

Interleukin-1 (IL-1) is a lymphokine released by macrophages in response to immunogenic stimulation. This polypeptide has been associated with a complex spectrum of biological activities. IL-1 is a primary immunostimulatory signal capable of inducing thymocyte proliferation via induction of interleukin-2 release, and stimulating proliferation and maturation of B-lymphocytes. In addition, IL-1 has been linked with prostaglandin production and induction of fever, and with promotion of wound healing. Reviews of the literature relating to IL-1 include Oppenheim et al., Immunol. Today 7:45 (1986), and Durum et al., Ann. Rev. Immunol. 3:263 (1985).

Human IL-1 activity resides in two distantly related proteins, herein designated IL-1 α and IL-1 β (March et al., Nature 315:641 (1985)). Both molecules are normally synthesized as larger precursors having molecular weights of about 30,000 daltons, which are subsequently proteolytically processed to yield mature forms having molecular weights of approximately 17,500 daltons.

Recently, cDNAs coding for both human IL-1 species

have been cloned and expressed in microorganisms. This achievement should enable production of sufficient quantities of IL-1 α and IL-1 β to permit therapeutic use. However, difficulties have been encountered in purification of active, nondenatured IL-1 species from cells capable of high level expression of the recombinant proteins. These difficulties have been attributed to the observation that the recombinant protein appears to be concentrated by the producing organisms in an insoluble form.

Gubler et al., J. Immun. 36:2492 (1986) reported a process for crude purification of IL-1 α expressed in E. coli. However, this process involved use of the denaturing agents guanidine hydrochloride and urea to enhance solubilization of the recombinant proteins.

The present invention provides a rapid, efficient acid-mediated recombinant protein solubilization process for isolating recombinant IL-1 from microbial cell cultures.

SUMMARY OF THE INVENTION

The present invention provides a process for purifying recombinant IL-1 from microbial cells, comprising:

(a) suspending the cells in an aqueous buffered medium having a pH from about 1 to about 5;

(b) disrupting the cells to provide an extract containing solubilized IL-1; and

(c) recovering solubilized IL-1 from the extract.

In a product aspect, the present invention provides purified recombinant IL-1 α having a specific activity greater than about 6.0×10^8 units per mg and endotoxin levels less than 100 pg endotoxin/ μ g rIL-1 α ; and purified recombinant IL-1 β having a specific activity greater than about 1.9×10^8 units per mg and endotoxin levels less than about 50 pg endotoxin/ μ g rIL-1 β , where endotoxin levels are measured by a limulus amebocyte lysate assay.

DETAILS OF THE INVENTION

The process of the present invention involves extraction of recombinant human IL-1 species from microbial cells under acid conditions. Acid extraction simultaneously solubilizes the IL-1 and precipitates the bulk of the microbial proteins, enabling recovery of IL-1 in supernatants of the acid extracts. Preliminary experiments have indicated that in E. coli, recombinant IL-1 is expressed in an insoluble form. In general, recombinant proteins can be solubilized with such chaotropic agents as guanidine or urea. In contrast, acid-mediated extraction avoids denaturing extractants, and allows purification to proceed directly from the initial extraction step to subsequent ion exchange procedures without solvent interference or protein denaturation. This process is thus ideally suited for scaling-up to provide commercially significant quantities of recombinant IL-1 α and IL-1 β .

As used herein, "interleukin-1", "recombinant interleukin-1", "IL-1" and "rIL-1" refer collectively to recombinant forms of IL-1 α and IL-1 β produced by microbial fermentation processes. In addition, the term comprehends proteins having amino acid sequences substantially identical to that of native mammalian forms of IL-1 α and IL-1 β , which possess biological activity in common with the native forms. Substantial identity of amino acid sequences means that the sequences are identical or differ by one or more amino acid alterations (deletions, additions, or substitutions) that do not cause an adverse functional dissimilarity between the synthetic protein and the native form.

Preferably, the acid-mediated extraction step for rIL-1 α is conducted in an aqueous buffered medium having a pH from about 2.0 to about 3.5, and most preferably, at a pH from about 2.6 to about 3.0. In the case of rIL-1 β , the acid extraction step is preferably conducted at a pH from about 3.5 to about 4.5, and most preferably, at a pH from

about 3.7 to about 4.1. As used herein, the term "microbial cells" means bacteria, particularly Escherichia coli, and yeast, e.g., Saccharomyces cerevisiae. In the process of this invention, cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

In a more complete process aspect, the acid extraction steps are coupled with subsequent chromatography in aqueous media. This part of the purification process preferably includes an initial ion exchange chromatography stage followed by affinity chromatography. The ion exchange stage comprises, in a preferred aspect, cation exchange chromatography followed by anion exchange chromatography.

Suitable cation exchange chromatography media include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred. The matrices can be acrylamide, agarose, dextran, cellulose or other ion exchange resins or substrates commonly employed in protein purification. A particularly useful material for cation exchange chromatography of rIL-1 α and rIL-1 β is Sulphopropyl Sephadex C-25 (Pharmacia Fine Chemicals, Uppsala, Sweden). When media containing sulfopropyl groups are employed, extracts containing rIL-1 species are applied at a pH of about 4.0, in a suitable buffer such as sodium citrate. rIL-1 species are bound by the ion exchanger, and can be eluted in more highly purified form by application of a weakly basic eluant, for example, 10 mM Tris-HCl, pH 8.1.

Suitable anion exchange chromatography media include various insoluble matrices comprising diethylaminoethyl(DEAE) or diethyl-(2-hydroxypropyl)aminoethyl (QAE) groups. DEAE groups are preferred. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. A particularly useful material for cation exchange chromatography of rIL-1 α and rIL-1 β is DEAE-Sephacel (Pharmacia). When media containing DEAE groups are

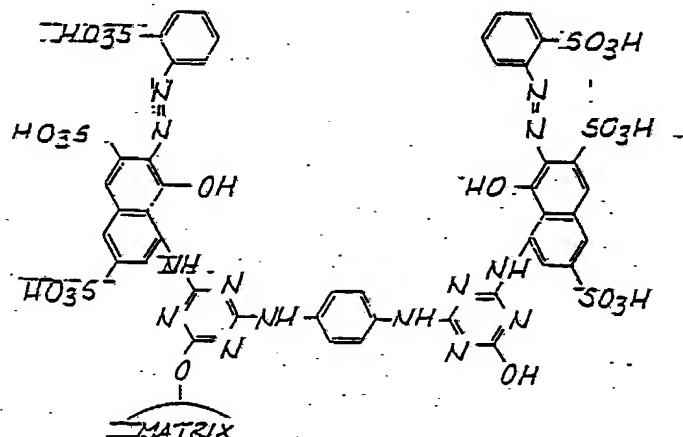
employed, extracts containing rIL-1 species are applied at a weakly basic pH. For example, the pooled rIL-1-containing fractions resulting from the previous cation exchange chromatography step (at a pH of about 8.1) can be applied directly in a suitable buffer such as Tris-HCl. rIL-1 species are bound by the anion exchange media, and can be eluted in more highly purified form by application of a salt gradient in the same buffer. It has been determined that rIL-1 α elutes from DEAE-Sephacel at 0.17-0.22 M NaCl, and rIL-1 β at 0.075-0.155 M NaCl. Thus, gradients ranging from 0 to 600 mM NaCl and 0 to 400 mM NaCl are useful in purifying rIL-1 α and rIL-1 β , respectively.

In its most preferred aspects, the invention provides a process including the foregoing extraction and ion-exchange chromatography procedures followed by affinity chromatography. For IL-1 α , affinity media comprising pendant phenyl glycidyl ether groups (or other groups providing a pendant phenyl nucleus attached by an ether or amide bridge to a suitable substrate) are preferred. Such media include, for example, Phenyl Sepharose CL-4B (Pharmacia) although other insoluble matrices, for example dextran or cellulose derivatives, could be employed. rIL-1 α is applied to such media in a solution containing about 0.5 to 0.7 M, and preferably, about 0.6 M ammonium sulfate, in a suitable buffer at a pH of about 8.1, and then eluted with a decreasing linear gradient of ammonium sulfate followed by buffer containing no salt. rIL-1 α elutes from Phenyl Sepharose CL-4B at about 0.25-0.10 M ammonium sulfate.

Thus, for IL-1 α , the most preferred process comprises suspending microbial cells having associated recombinant IL-1 α in an aqueous buffered medium having a pH from about 2.0 to about 3.5; disrupting the cells to extract solubilized IL-1 α ; applying the solubilized IL-1 α to cation exchange media at a pH from about 2.5 to about 5.0; eluting the IL-1 α from the cation exchange media at a pH from about 7.5 to about 9.0; applying the IL-1 α to anion exchange media

in a buffer having low osmolarity; eluting the IL-1 α in a gradient of increasing salt concentration; applying the IL-1 α , in a buffer containing 0.5 to 0.7 M ammonium sulfate, to media comprising pendant phenyl glycidyl ether groups, and eluting the IL-1 α in a gradient of decreasing ammonium sulfate concentration. The resulting fractions can be concentrated by a final chromatography step on media containing pendant sulfopropyl groups.

For the final affinity step for purifying IL-1 β , affinity media comprising pendant triazinyl red dye ligand groups are preferred. Such media include those having pendant groups



formed by conjugation of certain sulfonated naphtholine dye species known as Procion Red or "reactive red", with appropriate insoluble substrates. Commercially available materials meeting these criteria include Red Sepharose CL-6B (Pharmacia), reactive red-agarose (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) and Procion Red agarose (Bethesda Research Laboratories, Gaithersburg, Maryland U.S.A.) Preferably, pooled fractions containing rIL-1 β are applied to the dye-ligand media at a low ionic strength, e.g., less than 40 mM, in a suitable buffer such as 10 mM Tris-HCl. The media comprising bound rIL-1 β is then washed with additional application buffer, and the desired protein eluted in a linear gradient of increasing salt concentration, e.g., 0 to 1 M NaCl. rIL-1 β elutes from

Procion Red at about 0.36-0.46 M NaCl. The resulting fractions can be concentrated by a final chromatography step on media containing pendant sulfopropyl groups.

Thus, for rIL- β , the most preferred process
5 comprises suspending microbial cells having associated recombinant IL- β in an aqueous buffered medium having a pH from about 3.5 to about 4.5; disrupting the cells to extract solubilized IL- β ; applying the solubilized IL- β to cation exchange media at a pH from about 2.5 to about 5.0; eluting
10 the IL- β from the cation exchange media at a pH from about 7.5 to about 9.0; applying the IL- β to anion exchange media in a buffer having low ionic strength; eluting the IL- β in a gradient of increasing salt concentration;
applying the IL- β to media comprising pendant Procion Red
15 dye ligand groups; and eluting the IL- β in a gradient of increasing sodium chloride concentration.

1. Assays for IL-1 Activity and Endotoxin Levels

Progress of rIL- α or rIL- β purification can be
20 monitored by a thymocyte mitogenesis assay, which involves ascertaining the capacity of a sample to induce proliferation of thymocytes from CD-1 mice. In this assay, approximately 1×10^6 thymocytes, obtained from 10 to 12 week old CD-1 mice (Charles River Breeding Laboratories,
25 Wilmington, MA) are seeded in round bottom microplate wells (Corning Plastics, Corning, New York) in the presence of three-fold serial dilutions of the IL-1 containing fluid samples. The thymocytes are cultured in 150 μ l of Eagle's minimal essential medium (MEM) containing 50 U/ml
30 penicillin, 50 μ g/ml streptomycin, 2 mM glutamine, 0.2 mM gentamycin, 10 mM HEPES (N-2-Hydroxyethylpiperazine-N'-
-2-ETHANESULFONIC ACID) BUFFER, PH 7.4, TOGETHER WITH 4% w/v
human serum and 10^{-5} M 2-mercaptoethanol. The samples are
cultured for 72 hours at 37°C in an atmosphere of 5% CO₂ in
35 air. Thereafter, the cultures are pulsed for approximately 4 hours with 0.5 microcuries (μ Ci) of tritiated thymidine

(^3H -Tdr) after which the cultures are harvested onto glass fiber filter strips with the aid of a multiple-automated sample harvester. Details of this procedure are provided by Gillis et al., J. Immun. 120:2027 (1978) and in U. S. Patent 4,411,992.

In this assay, only the CD-1 cells cultured in the presence of IL-1 incorporate ^3H -Tdr in a dose-dependent manner. CD-1 cells cultured in the absence of IL-1 incorporate only background levels of radiolabel. IL-1 activity is calculated from the linear portion of the ^3H -Tdr incorporation data. Units of IL-1 activity are determined as the reciprocal dilution of a sample which generates 50% of maximal thymocyte ^3H -Tdr incorporation as compared to a laboratory standard.

Alternatively, IL-1 activity can be assayed by an IL-1 conversion assay, which relies upon the discovery that IL-1 converts an IL-2 nonproducer cell line, the murine tumor cell line LBRM-33-145, to an IL-2 producer. In this assay, LBRM-33-1A5 cells, (ATCC No. CRL-8079) are inactivated by addition of 50 $\mu\text{g/ml}$ mitomycin C and incubated for one hour at 37°C. 100 μl of the inactivated cells (5×10^5 cells/ml) are cultured in 96-well flat-bottomed plates in the presence of an equal volume of the mitogen, phytohemagglutinin (PHA, 1%) together with serial dilutions of samples. At hourly time intervals the existence of IL-2 activity generated by IL-1 triggered, mitomycin C-inhibited LBRM-33-1A5 cells is directly ascertained by adding 50 μl of IL-2 dependent CTLL-2 cells (8×10^4 cells/ml). The microwell cultures are then incubated for 20 additional hours followed by a 4 hour pulse with 0.5 μCi of ^3H -Tdr, and the resulting pulsed cultures assayed for thymidine incorporation as detailed above. Only the CTLL-2 cells added to wells previously contacted with IL-1 (thereby inducing IL-2 production in the inactivated LBRM cells) will incorporate radiolabel. This conversion assay is both more rapid and more sensitive than the

thymocyte mitogenesis assay.

Protein concentrations can be determined by any suitable method. However, the Bio-rad total protein assay (Bio-rad Laboratories, Richmond, California, USA) is preferred. SDS-PAGE can also be employed to monitor purification progress, substantially as described by Kronheim et al., J. Exp. Med. 161:490 (1985) or other suitable technique. Additional details regarding use of the IL-1 assays described above are disclosed by Conlon, J. Immun. 131:1280 (1983) and Kronheim et al., supra.

Endotoxin levels are conveniently assayed using a commercial kit available from Whittaker Bioproducts, Walkersville, Maryland, U.S.A., (Quantitative Chromogenic Lal QCL-1000) or its equivalent. This method uses a modified limulus amebocyte lysate and synthetic color-producing substrate to detect endotoxin chromogenically. Purified rIL-1 α and rIL-1 β are tested for presence of endotoxin at multiple dilutions. The assay is preferably performed shortly following completion of purification and prior to storage at -70°C. To minimize the possibility of bacterial contamination during the purification process itself, sterile buffers are preferably employed.

2. Production of rIL-1 α and rIL-1 β

A. Construction of bacterial expression vectors

Mature IL-1 α and IL-1 β can be expressed in E. coli under the control of the phage λ PL promoter and cI857ts thermolabile repressor. Expression plasmids for rIL-1 α and rIL-1 β production can be constructed from plasmid pPLc28 (ATCC 53082), plasmid pKK223-3 (available commercially from Pharmacia Fine Chemicals, Uppsala, Sweden) and plasmids containing IL-1 α clone 10A (March et al., supra; ATCC 39997) and IL-1 β clone IL-1-14 (ATCC 39925) as follows.

To create an expression vector for IL-1 α , a 3' portion of the IL-1 α gene, extending from Ser¹¹³

10

(nucleotides 337-339) to Ala²⁷¹ (nucleotides 811-813) is inserted into expression vector pPLc28. This is achieved by excising a 499 base pair AluI-NdeI fragment from the 10A clone, to which the following synthetic oligonucleotide

5 linker is joined:

AATTCTAGGATAATTA ATG TCA GCA CCT TTT AG
GATCCTATTAAT TAC AGT CGT GGA AAA TC

10 This linker includes AluI and EcoRI termini, a ribosome binding site, and ATG initiation codon in addition to the IL-1 α Ser¹¹³-Ser¹¹⁷ sequence. pPLc28 is then digested to completion with EcoRI and NdeI, and the resulting larger fragment isolated by agarose gel electrophoresis. The
15 linker, 10A clone, and plasmid fragments are then fused using T4 ligase, to provide an expression plasmid herein denoted pILP α . Additional details of the construction of pILP α can be found in the disclosure of copending, commonly assigned U. S. Patent Application Serial No. 721,765, the
20 disclosure of which is incorporated by reference herein.

The resulting construct is then employed to transform E. coli strain Δ H1 (ATCC 33767; Castellazi et al., Molec. gen. Genet. 117:211) to ampicillin resistance, using standard techniques. To express the plasmid-borne IL-1 α
25 gene, cultures of transformed Δ H1 are grown in L-broth without ampicillin. When the cultures reach an A₇₂₀ of about 0.5, the culture temperature is raised to about 42°C to promote derepression of the thermolabile PL promoter. After one hour at elevated temperature, cells are harvested
30 by centrifugation and flash-frozen in a dry-ice/methanol mixture. IL-1 α activity in cell extracts can be assayed by either the thymocyte mitogenesis or IL-1 conversion assays previously described. Details regarding purification procedures are provided in the following Examples.

35 rIL-1 β can be produced via construction of an plasmid, herein designated pILP β . This vector is assembled

from pILPc (March et al., supra), which is constructed by replacing the BamHI/EcoRI fragment of pKK223-3 with a Sau3A/EcoRI fragment from pPLc28 containing the λ PL promoter. This plasmid is digested to completion with EcoRI and PstI, and the largest fragment then ligated to a (1) a 669 base pair HpaII/PstI fragment from pIL-1-14 (ATCC 39925) containing the human IL-1 β gene (Ala¹¹⁷ to COOH terminus encodes active protein) and (2) the following EcoRI/HpaI synthetic oligonucleotide:

10

AATTCTAGGATAATTA ATG GCA CCT GTA CGA TCA CTG AAC TGC ACG CTC
GATCCTATTAAT TAC CGT GGA CAT GCT AGT GAC TTG ACG TGC GAG GC

Plasmid pILP β is then used to transform E. coli H1 or other cells containing a thermolabile repressor of PL transcription. Following growth to A₇₂₀ of about 0.5, expression of the rIL-1 β gene is obtained by heat induction as previously described. rIL-1 β activity, as in the case of rIL-1 α , can be identified using the thymocyte mitogenesis or IL-1 conversion assays cited above.

Examples 1 and 2: Protein Purification

The general purification scheme described in Examples 1 and 2, below, involved an initial acid extraction from cell pellets, followed by an SPS (Sulphopropyl Sephadex; Pharmacia) column chromatography step and elution from a DEAE-Sephacel (Pharmacia) column. Column fractions containing rIL-1 α were then applied to Phenyl Sepharose CL-4B (Pharmacia), while those containing rIL-1 β were applied to a Procion Red agarose (Bethesda Research Laboratories) column for final purification. Sterile buffers were used throughout the purification protocol to safeguard the product from contamination by endotoxin. Chromatography fractions were monitored for protein concentration by the Bio-rad total protein assay (Bio-rad Laboratories, Richmond, California, USA) and the progress of

purification evaluated by SDS-PAGE as described by Kronheim, J. Exp. Med. 161:490 (1985). IL-1 activity of column fractions was determined by the IL-1 assays previously referenced.

5 Experiments in which the pH of the initial extraction buffer was varied indicated that extraction of rIL-1 α from E. coli cell suspensions at pH 2.8 resulted in precipitation of significant quantities of contaminating proteins while enabling good recovery of rIL-1 α . Similar
10 experiments involving rIL-1 β indicated that pH 3.9 is optimal for precipitating unwanted proteins while solubilizing rIL-1 β . Thus, the optimal pH for this initial extraction step may vary between fermenter batches. For this reason, small-scale pilot runs may be employed to
15 determine optimal pH, particularly where large quantities of material are involved.

rIL-1 α and rIL-1 β were produced by growth and derepression of appropriate E. coli cells harboring high level thermolabile expression plasmids for rIL-1 α and
20 rIL-1 β . Cells were grown in a 10 liter Microferm Fermentor (New Brunswick) employing conditions of maximum aeration and vigorous agitation. An antifoaming agent (Antifoam A) was employed. Cultures were grown at 30°C in the super induction medium disclosed by Mott et al., Proc. Natl. Acad. Sci. USA 82:88 (1985) plus antibiotics, derepressed at a
25 cell density corresponding to $A_{600} = 0.05$ by elevating the temperature to 42°C and harvested 16 hours after the upward temperature shift. The cell mass was initially concentrated using the Pellicon Cassette System (Millipore). The cell
30 slurry was then centrifuged at 10,000 x g for 10 minutes at 4°C followed by rapid freezing of the cell pellets from 2.5 liter culture aliquots.

To achieve the initial acid extraction, cell
pellets obtained from 2.5 liters fermentation medium as
35 described above were suspended in about 20 ml 30 mM Tris-HCl buffer, pH 8, containing 5 mM EDTA and 1 mM

phenylmethanesulfonyl fluoride (PMSF). The resulting suspension was rapidly frozen in a dry ice/methanol bath and then thawed. Next, 200 ml of 30 mM sodium citrate buffer at pH 2.8 (rIL-1 α) or 3.9 (rIL-1 β), containing 5 mM EDTA and 5 250 μ g/ml lysozyme was added to the suspensions. The resulting acid suspensions were incubated for 60 minutes in a 37°C water bath. Following incubation, the extracts were rapidly frozen in a dry-ice/methanol bath, thawed, and then centrifuged at 4°C for 45 minutes at 38,000 x g. 10 Supernatants were then carefully decanted for use in the next purification step.

Extraction of rIL-1 α from the E. coli cell suspension at pH 2.8 resulted in the precipitation of 79% of the contaminating proteins and recovery of 62.5% of the 15 rIL-1 α . The resulting extract was applied to an SPS C-25 column at pH 4. The column had been preconditioned with 0.1% Triton X-100 (polyoxyethylene ether; Sigma Chemical Company, St. Louis, Missouri, USA) and 10% fetal calf serum to reduce nonspecific absorption of IL-1 activity to the 20 resin. First, the pH of the crude extract was raised to about 4.0 by addition of 1.0 N NaOH, and then the resulting solutions were applied to 20x2.5 cm columns containing SPS C-25, previously equilibrated with 10 mM sodium citrate, pH 4.0. The column was washed with 3 column volumes 10 mM 25 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 5.0, and desired protein eluted from the column with 10 mM Tris-HCl, pH 8.1. 10 ml fractions were collected, analyzed by SDS-PAGE, and stored at 4°C for additional purification. The elution with 10 mM Tris-HCl buffer at pH 8.1 resulted in 30 a pH rise after 3 column volumes and elution of the rIL-1 α , while 49% of the contaminating proteins remained bound to the gel.

Fractions containing IL-1 activity from the previous step were combined and then applied to a 15 x 2.5 35 cm column containing DEAE-Sephacel previously equilibrated with 10 mM Tris-HCl pH 8.1. Since rIL-1 α was eluted from

the SPS column in the equilibration buffer of the DEAE column, the SPS pool (200-250 ml) could be loaded directly onto the DEAE column, avoiding loss of activity by dialysis. The DEAE column was washed with five column volumes of the starting buffer and then eluted with linear gradient of 0 to 600 mM NaCl in 10 mM Tris-HCl, pH 8.1, in a total of two column volumes. 5 ml fractions were collected, analyzed by SDS-PAGE, and held at 4°C for further purification. rIL-1 α eluted from the column at 0.17-0.22 M NaCl. 66% of the contaminating proteins were eliminated in this step; 20% eluted earlier in the salt gradient and 46% remained bound to the gel after elution of rIL-1 α .

Fractions containing rIL-1 α were pooled (50-60 ml) and treated by addition of sufficient solid ammonium sulfate to provide a final concentration of 0.5 M. The resulting solution was then applied to a 30x2.5 cm column containing Phenyl Sepharose CL-4B, equilibrated with 10 mM Tris-HCl buffer, also 0.5 M in ammonium sulfate, at pH 8.1. The column was washed with 5 column volumes starting buffer, and eluted with a decreasing linear gradient of ammonium sulfate starting at 0.5 M and ending at 0 M in about 3 column volumes. Finally, the column was eluted with about 100 ml 10 mM Tris-HCl, pH 8.1. rIL-1 α eluted at about 0.25-0.10 M ammonium sulfate. 10 ml fractions were collected. PAGE of the fractions indicated that the Phenyl Sepharose step purified rIL-1 α to homogeneity. Those fractions containing rIL-1 α were pooled and concentrated by reapplication to SPS C-25 as described by Kronheim et al., supra. rIL-1 α was eluted using 10 mM phosphate buffered saline (PBS) at pH 8.2.

Purified rIL-1 α was stored at -70°C. The purified rIL-1 α contained only about 60 pg endotoxin per μ g IL-1 α , and exhibited a specific activity of 6.5×10^8 units per mg. Overall yield for fermentation and purification was about 16.8 mg purified rIL-1 α per liter culture.

Extraction of rIL-1 β from the E. coli cell

suspension at pH 3.9 resulted in precipitation of 69% of the contaminating proteins and recovery of 37.3% of the rIL-1 β activity. Extracts containing rIL-1 β were also applied to an SPS C-25 column which had been pretreated with 0.1% Triton X-100 (polyoxyethylene ether; Sigma Chemical Company, St. Louis, Missouri, USA) and 10% fetal calf serum. Protein was applied and eluted substantially as described for rIL-1 α , above. This SPS step removed 68% of the contaminating protein with good recovery of rIL-1 β .

Fractions containing IL-1 activity from the SPS step were combined and then applied to 15 x 2.5 cm columns containing DEAE-Sephacel previously equilibrated with 10 mM Tris-HCl pH 8.1. As described for rIL-1 α , above, the DEAE columns were washed with five column volumes of the starting buffer and then eluted with linear gradients of 0 to 400 mM NaCl in a total of two column volumes. rIL-1 β eluted from the DEAE column at 0.075 M to 0.155 M NaCl, absent 62% of the contaminating proteins.

Fractions containing rIL-1 β resulting from the DEAE column step were diluted 1:4 in 10 mM Tris-HCl buffer, pH 8.1, to reduce ionic strength to less than 40 mM, then applied to a 20x2.5 cm column containing Procion Red agarose previously equilibrated with 10 mM Tris-HCl buffer, pH 8.1. The column was washed with five column volumes of starting buffer, and then eluted with a linear gradient in five column volumes ranging from 0 to 1 M NaCl in 10 mM Tris-HCl buffer, pH 8.1. Fractions of 10 ml were collected, analyzed, and then concentrated as described for rIL-1 α , above. rIL-1 β eluted from the Procion Red column at 0.36-0.46 M NaCl.

Purified rIL-1 β thus obtained exhibited a specific activity of 1.95×10^8 units per mg, and contained only 36 pg endotoxin per μ g rIL-1 β . Overall yield of protein was about 85.2 mg purified protein per liter culture.

The results of the purification procedures described above are summarized in Table 1, below.

Table 1: Purification of Recombinant IL-1 α and IL-1 β

5		Total	Total	Yield (%)	Specific
		Protein	Activity		Activity
	<u>A. IL-1α:</u>	<u>(mg)</u>	<u>(U x 10¹⁰)</u>		<u>(U/mg x 10⁷)</u>
	Cell Suspension	4576	8.0	100	1.7
10	Cell Extract	968	5.0	62.5	5.1
	SPS Pool	493	5.0	62.5	5.1
	DEAE Sephacel pool	168	2.45	30.6	15.0
15	Phenyl Sepharose pool	42	2.73	34.1	65.0
	<u>B: IL-1β</u>				
	Cell suspension	10116	19.3	100	1.9
	Cell extract	3096	7.2	37.3	2.3
20	SPS pool	978	13.2	68.7	13.5
	DEAE-Sephacel pool	376	5.6	29.0	14.8
	Procion Red pool	213	4.16	21.6	19.5

25

30

35

CLAIMS

What is claimed is:

1. A process for purifying recombinant IL-1 from
5 microbial cells, comprising:

(a) suspending the cells in an aqueous buffered
medium having a pH from about 1 to about 5;

(b) disrupting the cells to provide an extract
containing solubilized IL-1; and

10 (c) recovering solubilized IL-1 from the extract.

2. A process according to Claim 1, wherein the
recombinant IL-1 is IL-1 α .

15 3. A process according to Claim 2, wherein the
cells are suspended in an aqueous buffered medium having a
pH from about 2.0 to about 3.5.

20 4. A process according to Claim 3, wherein the
cells are suspended in an aqueous buffered medium having a
pH from about 2.6 to about 3.0.

5. A process according to Claim 1, wherein the
recombinant IL-1 is IL-1 β .

25 6. A process according to Claim 5, wherein the
cells are suspended in an aqueous buffered medium having a
pH from about 3.5 to about 4.5.

30 7. A process according to Claim 6, wherein the
cells are suspended in an aqueous buffered medium having a
pH from about 3.7 to about 4.1.

35 8. A process according to Claim 1, wherein
solubilized IL-1 is recovered from the extract by
chromatography in aqueous media.

9. A process according to Claim 8, wherein the chromatography includes ion exchange chromatography.

5 10. A process according to Claim 9, wherein the chromatography includes affinity chromatography.

10 11. A process according to Claim 10, wherein the ion exchange chromatography includes a cation exchange step comprising applying the IL-1 to cation exchange media under acidic conditions, followed by elution under weakly basic conditions.

15 12. A process according to Claim 11, wherein the cation exchange step is followed by an anion exchange step comprising applying the IL-1 to anion exchange media in a low osmolarity buffer, followed by elution in a gradient of increasing salt concentration.

20 13. A process according to Claim 12, wherein the recombinant IL-1 is IL-1 α .

25 14. A process according to Claim 13, wherein the ion exchange chromatography is followed by an affinity chromatography step comprising application of IL-1 α in a buffer containing 0.4-0.6 M ammonium sulfate to media comprising pendant phenyl glycidyl ether groups, and elution in a gradient of decreasing ammonium sulfate concentration.

30 15. A process according to Claim 14, comprising:
(a) suspending microbial cells having associated recombinant IL-1 α in an aqueous buffered medium having a pH from about 2.0 to about 3.5;

35 (b) disrupting the cells to extract solubilized IL-1 α ;

(c) applying the solubilized IL-1 α to cation

19

exchange media at a pH from about 2.5 to about 5.0;

(d) eluting the IL-1 α from the cation exchange media at a pH from about 7.5 to about 9.0;

(e) applying the IL-1 α to anion exchange media in
5 a buffer having low osmolarity;

(f) eluting the IL-1 α in a gradient of increasing salt concentration;

(g) applying the IL-1 α in a buffer containing 0.5-0.7 M ammonium sulfate to media comprising pendant
10 phenyl glycidyl ether groups, and

(h) eluting the IL-1 α in a gradient of decreasing ammonium sulfate concentration.

16. Purified recombinant IL-1 α having a specific
15 activity greater than 6.0×10^8 units per mg and endotoxin levels less than 100 pg endotoxin/ μ g rIL-1 α as measured by a limulus amebocyte lysate assay.

17. A process according to Claim 12, wherein the
20 recombinant IL-1 is IL-1 β .

18. A process according to Claim 17, wherein the ion exchange chromatography is followed by a dye-ligand affinity chromatography step comprising application of IL-1 β
25 to media comprising pendant triazinyl red dye ligand groups, and elution of IL-1 β in a gradient of increasing salt concentration.

19. A process according to Claim 18, comprising:

30 (a) suspending microbial cells having associated recombinant IL-1 β in an aqueous buffered medium having a pH from about 3.5 to about 4.5;

(b) disrupting the cells to extract solubilized IL-1 β ;

35 (c) applying the solubilized IL-1 β to cation exchange media at a pH from about 2.5 to about 5.0;

20

(d) eluting the IL-1 β from the cation exchange media at a pH from about 7.5 to about 9.0;

(e) applying the IL-1 β to anion exchange media in a buffer having low osmolarity;

5 (f) eluting the IL-1 β in a gradient of increasing salt concentration;

(g) applying the IL-1 β to media comprising pendant triazinyl red dye ligand groups; and

10 (h) eluting the IL-1 β in a gradient of increasing NaCl concentration.

20. A process according to Claim 21, wherein the affinity chromatography media comprises pendant Procion Red dye ligand groups.

15 21. Purified recombinant IL-1 β having a specific activity greater than 1.90×10^8 units per mg and endotoxin levels less than 50 pg endotoxin/ μ g rIL-1 β as measured by a limulus amebocyte lysate assay.

20

25

30

35

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US87/02140**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ¹

According to International Patent Classification (IPC) or to both National Classification and IPC
INT. CL. C07K 15/00, 3/20, 3/28; C12P 21/00

US CL. 530/351, 412, 413, 414, 416, 417, 422; 435/68, 803

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System

Classification Symbols

US 530/351, 412, 413, 414, 416, 417, 422; 435/68, 803

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁵

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category ⁶	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	NATURE, VOL. 315, ISSUED JUNE 1985, CLONING, SEQUENCE AND EXPRESSION OF TWO DISTINCT HUMAN INTER LEUKIN-1 COMPLEMENTARY DNAs, (MARCH), PG. 641-47, (SEE PG. 644).	1-7, 16, 21
X	JOURNAL OF IMMUNOLOGY, VOL. 136, ISSUED APRIL 1986, RECOMBINANT HUMAN INTERLEUKIN 1 α - : PURIFICATION AND BIOLOGICAL CHARACTERIZATION", (GUBLER), P 2492 (SEE PG. 2492-93).	1-8, 13-17, 19-21
X	JOURNAL OF IMMUNOLOGY, VOL. 136, ISSUED MAY 1986, PURIFICATION TO HOMOGENEITY AND NH ₂ - TERMINAL AMINO ACID SEQUENCE OF A NOVEL INTERLEUKIN-1 SPECIES DERIVED FROM A HUMAN B-CELL LINE, (RIMSKY), PAGE 3304-10.	1-21
P,A	NUCLEIC ACID RESEARCH, VOL. 14, ISSUED OCTOBER 1986, "GENOMIC SEQUENCE FOR HUMAN PROINTERLEUKIN 1 BETA: POSSIBLE EVOLUTION FROM A REVERSE TRANSCRIBED PRO-INTERLEUKIN 1 ALPHA GENE", (CLARK) PAGE 7897-14.	1-7, 21
P,A	JOURNAL OF IMMUNOLOGY, VOL. 137, ISSUED DECEMBER 1986, "TWO INTER LEUKIN 1 GENES IN THE MOUSE. CLONING AND EXPRESSION OF THE CDNA FOR MURINE INTERLEUKIN 1 β ", (GRAY), PAGES 3644-48.	1-7, 16, 21

¹⁶ Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"S" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ¹

10 NOVEMBER 1987

International Searching Authority ¹

ISA/US

Date of Mailing of this International Search Report ²

23 DEC 1987

Signature of Authorized Officer ³

Garnette E. Draper
GARNETTE DRAPER

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ¹⁷ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	BIOCHEMISTRY, VOL. 25, ISSUED JUNE 1986, PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF TWO DISTINCT HUMAN INTERLEUKINS-1 FROM THE MYELOMONOCYTIC THP-1 CELL LINE," (MATSUSHIMA), PAGES 3424-29	1-21
A	JOURNAL EXPERIMENTAL MEDICINE, VOL. 162, ISSUED SEPTEMBER 1985, "AMINO ACID SEQUENCE ANALYSIS OF HUMAN INTERLEUKIN 1 (IL-1): EVIDENCE FOR BIOCHEMICALLY DISTINCT FORMS OF IL-1, (CAMERON), PAGES 790-801	1-21
P, A	JOURNAL CHROMATOGRAPHY, VOL. -387, -ISSUED JANUARY 1987, "CHROMATOFOCUSING OF N-TERMINALLY PROCESSED FORMS OF PROTEIN", (WINFIELD), PAGES 290-300.	1, 5-8, 17, 19-21
X	NUCLEIC ACIDS RESEARCH, VOL. 14, ISSUED APRIL 1986, "COMPLETE NUCLEOTIDE SEQUENCE OF THE GENE FOR HUMAN INTERLEUKIN 1 ALPHA", (FURUTANI), PAGES 3167-79.	1-7, 16